AGRICULTURAL MATERIALS

An Improved Method for Analysis of Cholecalciferol-Treated Baits

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A liquid extraction method is described that permits rapid determination of cholecalciferol (D_3) in rodenticidal grain baits. Purified D_3 was incubated for various time periods to produce pre-D_3. Response ratios (concentration/detector response) for various concentrations of pre-D_3 and D_3 in solutions permitted generation of a correction factor for direct quantitation of pre-D_3 in solutions with a pure D_3 standard. The method has equal precision and accuracy, yet is simpler and less time consuming and requires less solvents than widely accepted methods for extracting D_3 from grain baits. Recoveries from control oat baits fortified at 0.05 and 0.75 wt % were 100.9 and 98%, respectively. A standard curve for concentrations ranging from 6.4 to 204 μg/mL had an r^2 of 0.9999 and an intercept of zero and was linear and proportional. The method limit of detection was 2.0×10^{-4} wt % D_3.

Cholecalciferol (D_3) has found increased use as a toxicant for control of various rodent pests (1). Two commercially available rodenticides with D_3 as the toxicant are currently registered with the U.S. Environmental Protection Agency. One product is a pelletized mixture of a concentrated D_3 formulation mixed with ground cereal carrier. The other is a mixture of the concentrated formulation and squirrel oats. Both products have an active ingredient concentration of 0.075%.

Use of other toxicant concentrations is being studied at the National Wildlife Research Center (NWRC), where researchers apply a 7.5% D_3 oil-based concentrate formulation to steamed, slightly crimped rolled oats (oats). This research has allowed production of oat-based baits of various D_3 concentrations for laboratory and field research use and has necessitated development of analytical methods for determining D_3 concentrations in these baits.

High-performance liquid chromatography (HPLC) with UV detection provides specific and effective procedures for separating and quantitating D_3 and related isomers in feeds (2–7), but these procedures, as well as the official AOAC method (8), require saponification and high-temperature extraction prior to analysis. In a previously published method (9), the authors state that commercially prepared bait preparations are free of "other ingredients of interest," and that extensive cleanup procedures are probably unnecessary. That method (9) involved a 4 h extraction in a Goldfisch apparatus with 0.1% butylated hydroxytoluene/acetonitrile (BHT) followed by HPLC analysis. Recovery was 98 ± 2.7%, but it was not easily replicated by other laboratories, perhaps because of the relative insolubility of D_3 in acetonitrile compared with other solvents. Hexane–isopropyl alcohol mixtures readily dissolve D_3 and related compounds and have been extremely useful as extraction solvents and LC mobile phases (10). However, even higher solubilities for both D_3 and the inert matrix components in commercial rodenticides are possible with isooctane (11).

The primary difficulty with any high-temperature saponification or extraction involving D_3 is the well-documented thermally induced isomerization of D_3 to pre-D_3, the only isomer or degradation product produced by D_3 incubation at ≤100°C (12). Both D_3 and pre-D_3 are biologically active, and their concentrations must be summed to express the total D_3 activity in a given sample. While this isomerization can be controlled by equal treatment of standard D_3 solutions, a short-duration room-temperature extraction that results in little or no isomerization would be more straightforward. Such a technique is described in this report.

Experimental

Reagents

(a) *Extraction solvent and standard diluent.*—Isooctane.

(b) *2,6-Di-tert-butyl-4-methylphenol.*—99% (BHT).

(c) *D_3 standard solutions.*—D_3 is photosensitive, and its solutions should be protected from light and kept at temperatures below 4°C to reduce isomerization. (*1*) *Concentrated fortification standard.*—Accurately weigh 1.00 g D_3 (99.5% purity; Aldrich Chemical Co., Milwaukee, WI) and dissolve in 10.00 mL diluent to make a 100.0 mg/mL solution. (2) *Concentrated analytical standard.*—Accurately weigh 12.5 mg D_3 and dissolve in 25.00 mL diluent to make a 500 μg/mL concentrated standard. (*3*) *Working standard (high level).*—0.75% D_3 oat bait: Transfer 3.00 mL of the 500 μg/mL concentrated analytical standard to a 10 mL volumetric flask. Add diluent to volume to make a 150 μg/mL

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working standard. The standard concentration is equivalent to 100% analyte recovery from 0.75% D_3 baits extracted by this method. *(4) Working standard (low level).*—0.05% D_3 oat bait: Transfer 1.00 mL of the 150 μg/mL working standard to a 10 mL volumetric flask. Add diluent to volume to make a 15 μg/mL working standard. The standard concentration is equivalent to 100% analyte recovery from 0.075% D_3 baits and is used to quantitate 0.05 and 0.1% D_3 baits.

Apparatus

(**a**) *Calibrated 50 mL graduated polypropylene centrifuge tubes.*

(**b**) *Calibrated 10 mL glass centrifuge tubes with Teflon-lined screw caps.*

(**c**) *Eberbach horizontal shaker with 2⅜" stroke.*

(**d**) *Sonicator.*

(**e**) *Coffee mill.*

(**f**) *Soxhlet apparatus and thimbles (25 × 90 mm).*

(**g**) *Mantle heater.*

(**h**) *Liquid chromatograph.*—Hewlett-Packard 1090 liquid chromatograph (Hewlett-Packard Co., Sunnyvale, CA) equipped with a diode array detector calibrated and set at 265 nm (UV).

(**i**) *Analytical column.*—Keystone Hypersil Silica, 3 μm, 120 Å, 4.6 mm × 25 cm (Keystone Scientific, Inc., Bellefonte, PA). Guard column is equivalent to the analytical column.

(**j**) *Mobile phase.*—97% *n*-hexane–3% isopropyl alcohol, 1.0 mL/min flow rate.

Extraction of Oat Baits

Several grams of oat bait were finely ground in a coffee mill. A 1 g sample was transferred to a 50 mL polypropylene centrifuge tube, followed by 25 mL extraction solvent. The tube was capped tightly, wrapped in aluminum foil to exclude light, and shaken horizontally at high speed for 15 min to extract D_3. The tube was then centrifuged at ca 2500 rpm for 5 min, and the supernatant was decanted into a 50 mL volumetric flask. A second 25 mL extraction solvent was added to the tube, and the tube was rewrapped, shaken, and centrifuged as previously described. Extracts were combined in the 50 mL volumetric flask, which had been protected from light during the second extraction. With fresh solvent, the total extract volume was brought to 50 mL and mixed. A portion of the extract was then filtered through a 0.45 μm Teflon syringe filter into an amber LC vial and immediately analyzed. For commercial 0.075% D_3 baits, the final extract concentration is ca 15 μg/mL.

Pre-D_3 Correction Factor

Although pre-D_3 is not produced in significant quantities with this procedure, bait samples could contain significant amounts of pre-D_3 as a formulation by-product or as a consequence of prolonged exposure. Because a pre-D_3 analytical standard is not commercially available, it is necessary to assess pre-D_3 concentrations by using a D_3 standard. This assessment was accomplished by developing a correction factor, which is the ratio of the molar extinction coefficients of pre-D_3 and D_3 at 265 nm (the optimum D_3 absorbance wavelength). To obtain this correction factor, increasing pre-D_3/D_3 ratios were formed by high-temperature incubation of a D_3 standard solution at increasing time intervals. Thirty 10 mL volume-calibrated centrifuge tubes were divided into 5 sets of 6 tubes each. Each set was incubated in a boiling water bath for one of 4 time periods (16, 30, 60, and 150 min). Prior to incubation, a 100.1 μg/mL D_3 standard (200 mL) was freshly prepared in isooctane and ca 2 mL was introduced into each of 6 amber LC vials to be used as unincubated standards during analysis. The LC vials were stored in a freezer at –16°C until needed. Then, 5.00 mL of the standard solution was pipetted into each centrifuge tube, the tube was capped (caps contained a Teflon-faced insert to permit sealing), the actual solution volume in the tube was recorded, and the tube was kept at –16°C until all tubes had been treated to minimize premature isomerization of D_3. Following pipetting of the standard solution into all centrifuge tubes, tubes were stored for 30 min to ensure temperature equalization.

Table 1. Pre-D_3 correction factors for various incubation times

	Correction factor		
Incubation time, min	Mean	SD	*n*
16	0.4410	0.01	6
30	0.4449	0.012	6
60	0.4424	0.02	6
150	0.4294	0.1	6

Each set of tubes was removed from the freezer just immediately prior to incubation. The tubes were placed in a boiling water bath (ca 94°C) and incubated for the appropriate time. Then, all tubes were removed from the bath, placed in an ice-water bath to suspend isomerization, and placed into a room-temperature water bath (22°C) for 5 min. Then the volumes in the tubes were checked to determine if solvent loss had occurred. Two tubes required slight (<100 μL) solvent addition to correct volume. Tubes were inverted thoroughly to mix, and ca 2 mL was removed from each tube and transferred to the corresponding amber LC vial for quantitation of D_3 and pre-D_3 by HPLC. The number of moles of pre-D_3 formed was calculated as the difference in moles of D_3 before and after incubation. Individual response factors (response/mass) for D_3 and pre-D_3 were then determined. The pre-D_3 correction factor is the mean of pre-D_3/D_3 response factors at various pre-D_3/D_3 concentrations.

Method Validation

(**a**) *Preparation of control oat bait.*—Commercial baits typically contain 0.075 or 0.1% D_3. Experimental baits evaluated by NWRC and available for potential registration contain 0.1 and 0.5% D_3. To develop a method suitable for these products, we prepared 0.05 and 0.75% D_3 baits for validation. Control bait formulations (containing no D_3) were prepared by adding the amount of oil-based concentrated control formula-

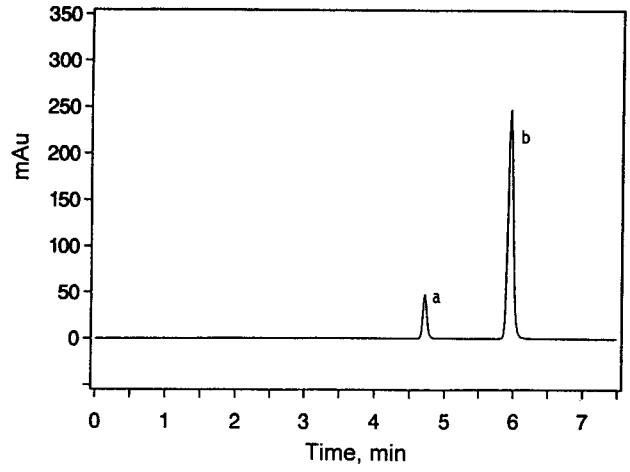

Figure 1. Chromatogram of a 100 μg/mL D_3 standard boiled to produce pre-D_3: a, pre-D_3; b, D_3.

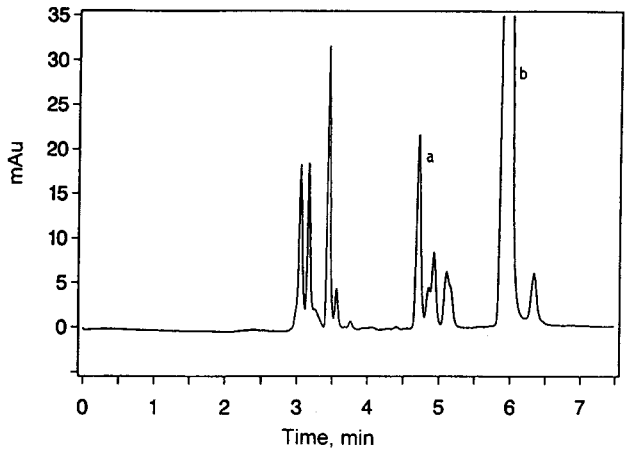

Figure 3. Enlarged chromatogram of a 0.75% D_3 bait: a, pre-D_3; b, D_3.

tion (CCF) contained in 0.05 and 0.75% baits. The CCF was gently warmed and applied to oats while mixing. After CCF addition, each control bait formulation was mixed for 5 min more to ensure homogeneous coating of the oats by the CCF. The control bait formulation was then refrigerated until used.

(b) *Bias and repeatability.*—One gram of the 0.05% CCF-treated oats was weighed into each of 7 polypropylene centrifuge tubes. The 100 mg/mL concentrated fortification standard was brought to room temperature in a water bath and mixed thoroughly. With a calibrated glass syringe, 5 μL of the fortification standard was added to the CCF-treated oats, and the mixture was dried immediately under an N_2 stream. All remaining samples were similarly fortified.

The procedure was repeated, adding 75 μL of the 100 mg/mL concentrated fortification standard to each of seven 1 g replicates of the 0.75% CCF-treated oat baits. All 0.05 and 0.75% fortified replicates were then extracted and analyzed by the procedure described.

(c) *Selectivity and method limit of detection.*—To assess matrix interferences, three 1 g samples of 0.75% CCF-treated oat baits were extracted, diluted, and analyzed by HPLC. The 0.75% control baits were prepared with 15 times more CCF than the 0.05% control baits, resulting in a much larger potential for interferences from CCF. The method limit of detection (MLOD) was defined as the concentration of D_3 in a bait required to generate a signal 3 times the baseline noise (measured peak to peak) in the control bait chromatograms at the retention time of D_3.

MLOD was estimated from a 1 g sample of oats fortified with CCF (0.75%) and D_3 (0.0025%), and chromatographic responses were compared with those obtained from the 3 control baits used to assess matrix interference.

Soxhlet Extraction

Soxhlet extraction of D_3 is used by both AOAC INTERNATIONAL for various feed matrixes and by Bell Laboratories, a producer of commercially available grain-based D_3 rodent baits. Soxhlet extraction was compared with liquid extraction by performing both procedures on a series of grain baits at the NWRC. A 0.075% D_3 on crimped whole oats was produced and analyzed at the NWRC. Experimental oat baits with nominal concentrations of 0.0, 0.0375,

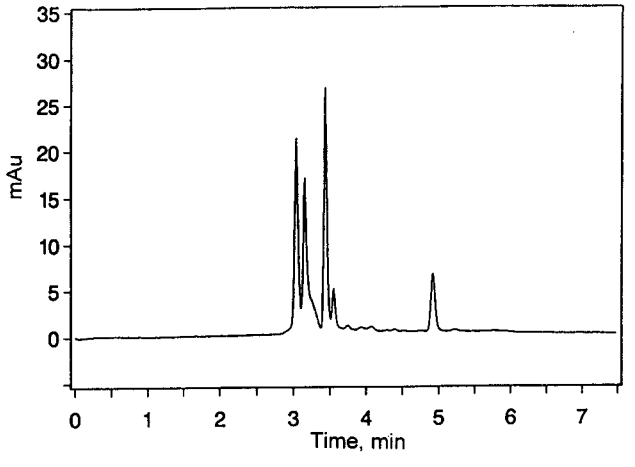

Figure 2. Enlarged chromatogram of a 0.75% D_3-equivalent control bait.

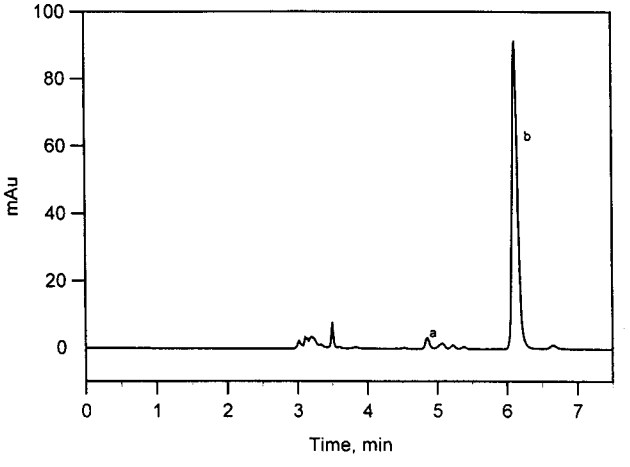

Figure 4. Chromatogram of a commercially available 0.075% D_3 bait: a, pre-D_3; b, D_3.

0.05, 0.075, and 0.15% were prepared, analyzed, and provided by Bell Laboratories.

The soxhlet extraction procedure of Bell Laboratories was modified to accommodate the limited amounts of available baits. Triplicate 5 g samples of finely ground bait were accurately weighed and transferred to separate extraction thimbles, which were each placed in individual soxhlet extraction apparatus. A D_3 soxhlet extraction standard was prepared by weighing the amount of D_3 nominally contained in 5 g of the concurrently run bait into a thimble and placed into a fourth apparatus. Approximately 1.0 mg BHT was added to each of four 250 mL round-bottom flasks, followed by ca 230 mL extraction solvent, and 5–6 boiling chips. Each apparatus was assembled, and the mantle heater control was set to provide ca 100°C to boil the solvent. After the D_3 standard and bait samples had been extracted for 4 h, each extract was allowed to cool, quantitatively transferred to a 250 mL volumetric flask, brought to volume with extraction solvent, and thoroughly mixed. Approximately 2 mL of each extract was transferred to a 3 mL syringe and filtered through a 0.45 μm Teflon syringe filter directly into a 2 mL amber LC vial, which was then capped, and the sample was immediately analyzed. A second D_3 nonextracted standard was prepared in a 10 mL volumetric flask to approximate the preextraction concentration of the soxhlet-extracted D_3 standard. It was stored in the dark at <0°C to minimize isomerization until analyzed with the extracted standard and bait samples.

Sample Calculation

The D_3 content in soxhlet-extracted bait samples was calculated with 3 formulas to compare results.

(**a**) *NWRC method*:

$$D_3,\ \text{wt}\ \% = \frac{A_{D_3}}{A_{\text{std}}} \times \frac{50.0\ \text{mL}}{\text{sample wt (g)}} \times \frac{1.00\ \text{g}}{1.00 \times 10^6\ \mu\text{g}} \times 100$$

$$\text{pre-}D_3,\ \text{wt}\ \% = \frac{A_{\text{pre-}D_3}}{0.44 \times A_{\text{std}}} \times \frac{50.0\ \text{mL}}{\text{sample wt (g)}} \times \frac{1.00\ \text{g}}{1.00 \times 10^6\ \mu\text{g}} \times 100$$

where A_{D_3}, $A_{\text{pre-}D_3}$, and A_{std} are the chromatographic peak responses from the sample D_3, pre-D_3, and standard D_3, respectively; C_{std} is the D_3 working standard concentration (15 or 150 μg/mL); 50.0 mL is the volume of the volumetric flask; sample wt is the weight of the ground bait sample (ca 1 g); 0.44 is the correction factor for the difference in the extinction coefficients of pre-D_3 and D_3.

(**b**) *Bell Laboratories method (modified)*:

$$D_3,\ \text{wt}\ \% = \frac{\text{std wt (g)}}{\text{sample wt (g)}} \times \frac{A_{D_3} + A_{\text{pre-}D_3}}{A_{\text{std }D_3} + A_{\text{std pre-}D_3}} \times \frac{V_{\text{std}}}{V_{\text{samp}}} \times 100$$

where std wt is the weight of D_3 standard extracted, sample wt is the weight of ground bait extracted (ca 5 g), $A_{D_3} + A_{\text{pre-}D_3}$ are the combined areas of the sample D_3 and pre-D_3 chromatographic responses taken at 254 nm, $A_{\text{std }D_3} + A_{\text{std pre-}D_3}$ are the combined areas of the standard D_3 and pre-D_3 chromatographic area responses taken at 254 nm, and V_{std} and V_{samp} are the final post-extraction volumes of the standard and sample solutions, respectively.

(**c**) *AOAC Official Method* ***982.29.***—Modified to yield wt %:

$$D_3,\ \text{wt}\ \% = \frac{1.25 \times P \times W' \times V \times 1\ \text{mg}}{P' \times W \times V' \times 1000\ \mu\text{g}} \times 100$$

where 1.25 is the correction ratio for pre-D_3 formed during extraction, P is the peak height of D_3 in the sample solution, W' is the weight of the reference standard (mg), V is the volume of sample solution (mL), P' is the peak height of D_3 in the standard solution, W is the sample weight (g), and V' is the volume of reference standard solution (mL).

Calculations according to the NWRC method were performed with data collected at 265 nm, while the calculations according to the Bell Laboratories and AOAC methods were performed with data taken simultaneously at 254 nm.

Table 2. Recoveries from laboratory-fortified D_3 oat baits

	Recovery, %	
Sample	0.05% Bait	0.75% Bait
1	100.2	98.2
2	100.3	98.0
3	101.2	98.1
4	101.2	97.5
5	101.2	97.9
6	101.3	98.2
7	100.9	97.9
	$\bar{x}$ = 100.9	$\bar{x}$ = 98
	SD = 0.46	SD = 0.24
	CV = 0.46%	CV = 0.24%

Results and Discussion

Pre-D_3 Correction Factor

Results of the D_3 incubation experiment are shown in Table 1. To determine sample response factors, molar concentrations of D_3 in all postincubation samples were calculated. Molar concentrations of pre-D_3 in each sample were derived by subtracting the corresponding D_3 molarity from that of controls. Group mean pre-D_3/D_3 ratios for the 4 incubation time

Table 3. D_3 concentrations in grain bait

Extraction method	Calculation method	D_3 found, %, in indicated sample									
		NWRC, 0.075%	Bell, 0.0%	Bell, 0.0375%	Bell, 0.05%	Bell, 0.075%	Bell, 0.15%	Bell pellet 40015	Bell pellet 40612	Bell mouse seed 40610	Bell mouse seed 39941
Soxhlet by Bell	Bell	ND[a]	0.0	0.0418	0.064	0.096	0.19	0.075[b]	0.075[b]	0.1[b]	0.1[b]
NWRC	NWRC	0.0796	0.0	0.0320	0.0499	0.0807	0.180	0.0875	0.0744	0.102	0.0919
		(0.001)[c]		(0.0003)	(0.001)	(0.0007)	(0.004)	(0.0005)	(0.0009)	(0.0008)	(0.001)
		6[d]	6[d]	6[d]	6[d]	4[d]	5[d]	6[d]	6[d]	6[d]	6[d]
Soxhlet by NWRC	Bell	0.08459	0.0	0.0370	0.0496	ND	0.163	ND	ND	ND	ND
		(0.0009)		(0.004)	(0.004)		(0.004)				
		3[d]	3[d]	3[d]	4[d]		3[d]				
	NWRC	0.08445	0.0	0.0358	0.0479	0.0751	0.162	ND	ND	ND	ND
		(0.0004)		(0.002)	(0.003)	(0.001)	(0.002)				
		3[d]	3[d]	3[d]	4[d]	3[d]	3[d]				
	AOAC	0.07892	0.0	0.0334	0.0476	ND	0.153	ND	ND	ND	ND
		(0.0005)		(0.002)	(0.005)		(0.0007)				
		3[d]	3[d]	3[d]	4[d]		3[d]				

[a] ND = no data.
[b] Nominal concentrations.
[c] Values in parentheses are standard deviations.
[d] Number of sample replicates.

periods are given, along with standard deviations (SDs) and number of replicates. The total mean ratio for all time periods was 0.44. This value was used as the correction factor for pre-D_3 calculations using a D_3 standard. Earlier studies reported correction ratios of 0.586 (4) and 2.45 (1/0.408; 13), but the overall approach to generating these correction factors was disputed by other authors (12, 14), who stated that such ratios are variable and condition dependent. In all these studies, UV detection of pre-D_3 and D_3 was performed at 254 nm. As stated previously, the absorbance maximum of D_3 occurs at 265 nm, while that of pre-D_3 occurs at 260 nm (15, 16). For both compounds, but especially for D_3, 254 nm is on the steep upslope of the absorbance spectrum, and estimates made at this wavelength are prone to error. By optimizing D_3 detection at 265 nm, this problem is eliminated, permitting quantitation of pre-D_3 in various HPLC systems with only a D_3 standard. However, while the approach taken to generate the correction factor is sound, interlaboratory variations in instrumentation may make it advisable to confirm the correction factor as part of an overall method validation strategy.

Response Linearity

Prior to analysis of the D_3-fortified control oat baits, 2 separate sets of 5 D_3 standards were prepared, ranging in concentration from 6.4 to 204 μg/mL, corresponding to bait D_3 concentrations of 0.032 to 1.02%. All standards were sampled in duplicate. The linear regression analysis of chromatographic peak area (y-axis) versus D_3 concentration (x-axis) generated a slope of 16.661, a y-intercept of –0.20 (HO: $y_{int} = 0$; $p = 0.9379$), and an r^2 of 0.9999. Response ratios (concentration/response) were unchanged throughout the D_3 concentration range, with a mean value of 0.06 ($\pm 3.4 \times 10^{-4}$) and a CV of 0.57%. In addition, a log versus log regression of the same data yielded a slope of 0.9993. These results indicate a highly linear, directly proportional relationship between response and D_3 concentration. They demonstrate that use of a single-point calibration standard is appropriate to assess oat bait D_3 concentrations in typical 0.075 and 0.1% D_3 bait products, as well as in any likely future experimental baits.

Selectivity and Method Limit of Detection

No chromatographic interferences were observed in any of the CCF-treated oat samples at the retention times of pre-D_3 or D_3. Figure 1 is a chromatogram of a 100 μg/mL D_3 standard (5.9 min) boiled for 30 min to create pre-D_3 (4.7 min). The MLOD was calculated as a wt % bait equivalent of 2×10^{-4}%. Chromatograms of a control 0.75% bait and a 0.75% D_3 bait are shown in Figures 2 and 3, respectively. These baits represent worst case concentrations for both the CCF and D_3. A typical chromatogram from the analysis of a commercially produced 0.075% D_3 bait product is presented in Figure 4.

Bias and Repeatability

D_3 recoveries from 0.05 and 0.75% laboratory-fortified D_3 oat baits are shown in Table 2. Mean D_3 recoveries were at least 98% for both concentrations, and the low CVs indicate a highly repeatable extraction procedure.

Sample Extraction and Calculation of D_3 Concentration

A comparison of D_3 concentrations found in various D_3 grain bait products using the NWRC liquid extraction method and the soxhlet method done at NWRC and Bell Laboratories is presented in Table 3. D_3 concentrations were calculated using each of the 3 formulas previously described. Some differences were observed between Bell's soxhlet extraction and the NWRC liquid extraction, especially at lower concentrations of 0.0375 and 0.05%. Soxhlet extraction of the same baits done at NWRC yielded results very similar to those found by NWRC liquid extraction. Between-laboratory differences in soxhlet extraction results may have been due to differences in sample sizes (5 g versus 20 g).

Method reproducibility was excellent for analysis of commercial products (Table 3), with CVs (not shown) ranging from 0.57 to 1.2%. Additionally, the NWRC calculation using the 0.44 correction factor gave concentrations very similar to those obtained by the Bell calculation, which sums the chromatographic responses from the pre-D_3 and D_3 peaks within each sample (11). The AOAC calculation yielded D_3 concentrations similar to those derived from the other methods.

Conclusions

The extraction described in this report is simple, reliable, and repeatable, with a very low limit of detection. It requires a small sample size (1 g), making it useful for residue detection in small field samples. It also eliminates the need for tedious, potentially explosive, and solvent- and time-consuming soxhlet and Goldfisch procedures. This method offers the additional advantage of increased sample throughput without large quantities of extraction glassware and associated apparatus. Furthermore, because the method produces little or no pre-D_3 through heat-induced isomerization, the results indicate the true concentrations of D_3 and pre-D_3 in actual samples. The calculation using the derived 0.44 correction factor allows direct quantitation of pre-D_3 by use of a pure D_3 standard, minimizing the expense involved in the larger D_3 standard masses required for soxhlet procedures. Data from the method are comparable with those from soxhlet-extracted bait samples over a wide range of concentrations.

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